# Recent improvements in the use of CHT™ ceramic hydroxyapatite for removal of aggregates from monoclonal Antibodies

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# *Why use CHT for MAb Purification?*

| Contaminant | Method | Clearance |
|---|---|---|
| Aggregates | HPSEC | 1-2 logs |
| Protein A | Cygnus | 1-2 logs |
| CHOP | ELISA | 2 logs |
| DNA | Picogreen | > 3 logs |
| Endotoxin | LAL (chromo) | > 4 logs |
| aMULV<br>xMULV<br>MVM<br>PPV | Infectivity<br>Infectivity<br>Infectivity<br>Infectivity | > 4 logs<br>> 3 logs<br>2 logs<br>> 1 log |

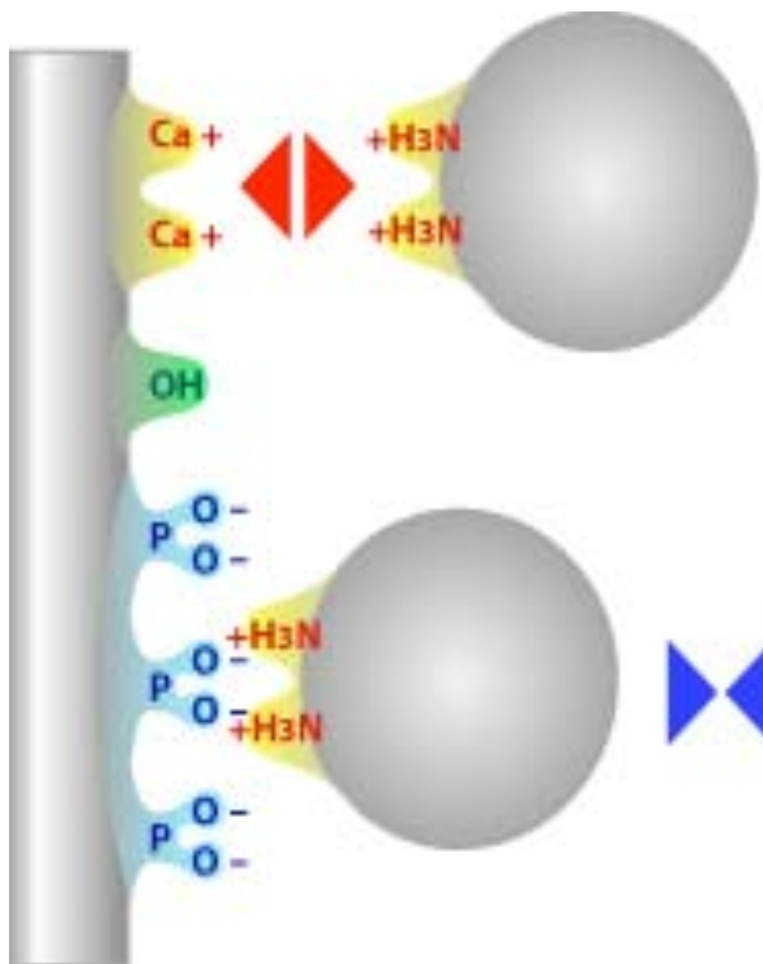
NaCl gradients at constant phosphate concentration on CHT type I 40 µm

# What is CHT?

Hydroxyapatite is a crystalline mineral of calcium and phosphate with the structural formula $Ca_{10}(PO_4)_6(OH)_2$

Hexagonal cross section nanocrystals (~10 x 100 nm) are agglomerated into porous spheres, then fused at high temperature to form a stable ceramic chromatography adsorbent (CHT).

# How CHT works



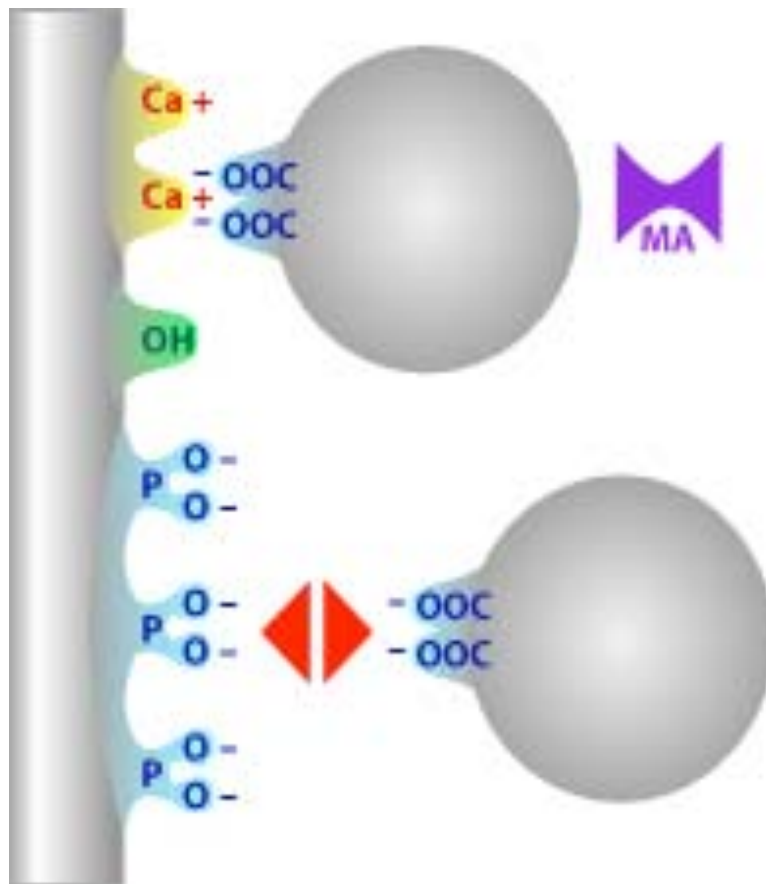

**Amino residues**

Classical cation exchange

Dissociate with neutral salts like sodium chloride or with buffering salts like phosphate.

Weaken or dissociate with increasing pH

# How CHT works




**Carboxyl clusters**

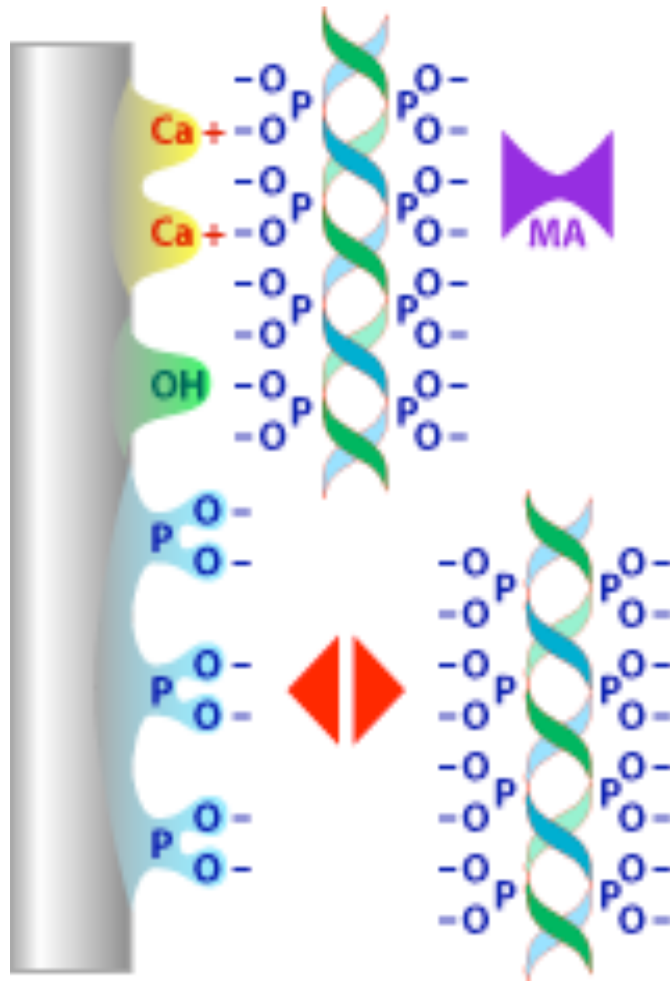
Calcium chelation modulated by ion exclusion

15-60x stronger than ionic interactions alone

Will not dissociate at any concentration of sodium chloride

Dissociation requires phosphate

# How CHT works




**Phosphoryl residues**

Calcium coordination modulated by ion exclusion

15-60x stronger than ionic interactions alone

NaCl causes *stronger* DNA binding by suppressing charge repulsion between phosphates

Dissociate with phosphate

# How CHT works with IgG

Most published applications report the use of phosphate gradients for IgG purification.

Phosphate gradients simultaneously dissociate calcium affinity and cation exchange, but do not permit independent control of the two mechanisms.

Recent experience indicates that more effective contaminant clearance can be achieved with sodium chloride gradients at constant low phosphate concentrations.

# How CHT works with IgG

Most IgG monoclonals have weak affinity for CHT calcium but fairly strong charge interactions with CHT phosphates.

Setting a constant low level of phosphate suspends weak calcium affinity interactions but leaves strong ionic interactions intact. A sodium chloride gradient can then dissociate ionic bonds. Monomeric IgG elutes first. Aggregates elute later.

Contaminants with a strong calcium affinity remain bound to the column until it is cleaned with concentrated phosphate. These include leached protein A-IgG complexes and phosphorylated contaminants such as DNA, endotoxin, and lipid enveloped viruses.

# *How CHT works with IgG*

Chloride gradients are more effective than phosphate gradients

| Parameter | Chloride | Phosphate |
|---|---|---|
| Monomer recov. | 82% | 78% |
| Aggregate | < 1% | < 1% |
| Protein A | < 1 ppm | < 1 ppm |
| CHOP | <12 ppm | < 72 ppm |
| DNA | < 1 ppm | < 7 ppm |
| Endotoxin | < 0.1 EU/mL | < 5.0 EU/mL |

Human/mouse IgG1 chimera

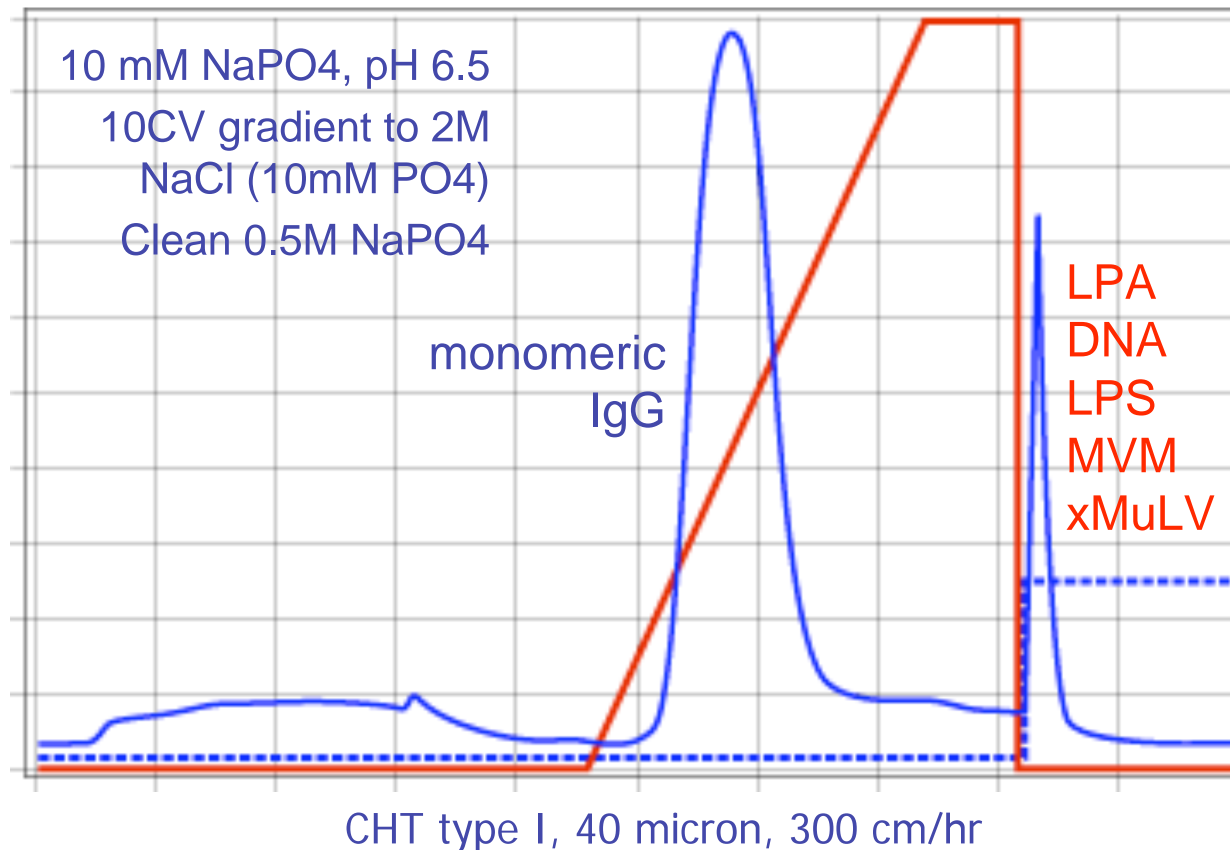
# *How CHT works with IgG*

Eluting CHT with a sodium chloride gradient at a fixed low concentration of phosphate has provided excellent reduction of aggregates, leached protein A, HCP, DNA, endotoxin, and virus with every monoclonal antibody evaluated to date. *This includes rat, guinea pig, mouse, chimeric, and human IgG monoclonals from various subclasses*.

The consistency of elution behavior among these diverse samples suggests that applicability of this approach may be essentially universal.

# How CHT works with IgG

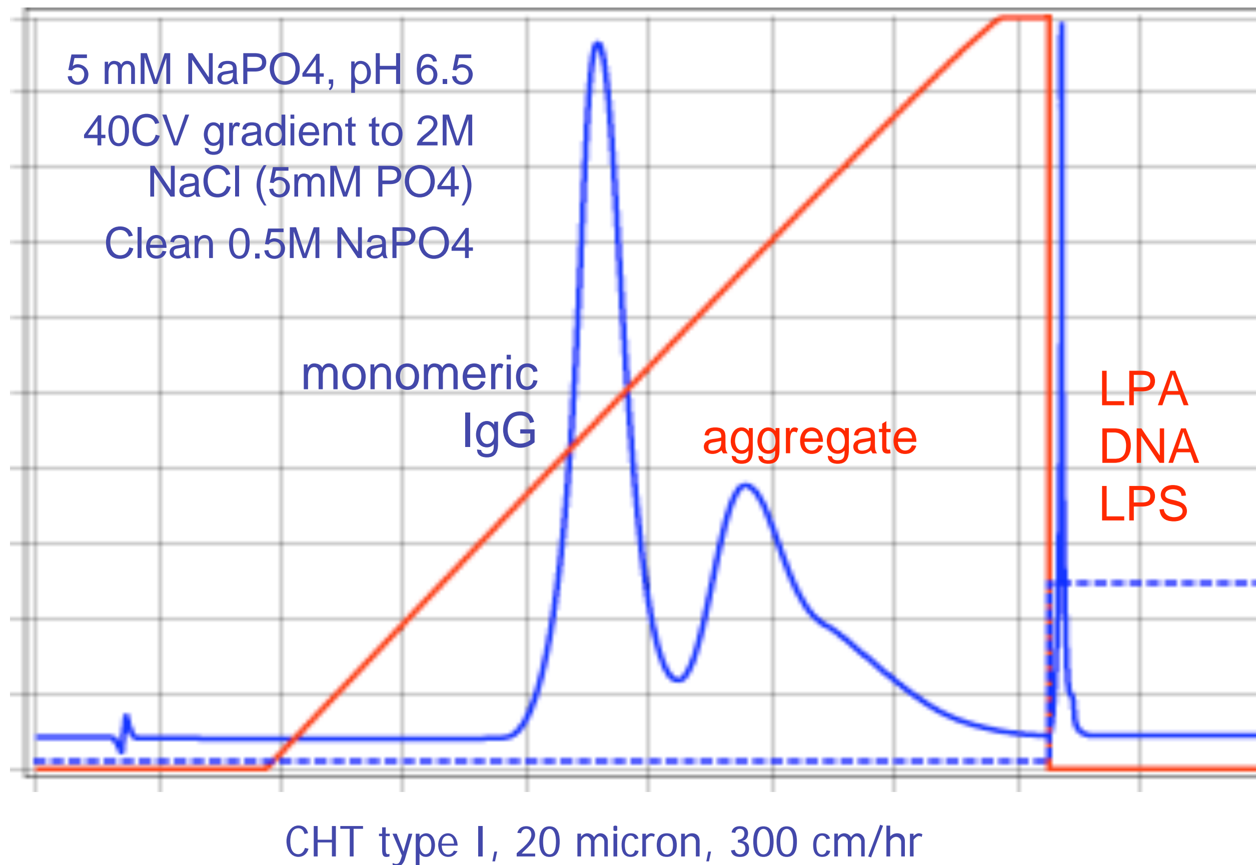
protein A purified human IgG1




CHT type I, 40 micron, 300 cm/hr

# How CHT works with IgG

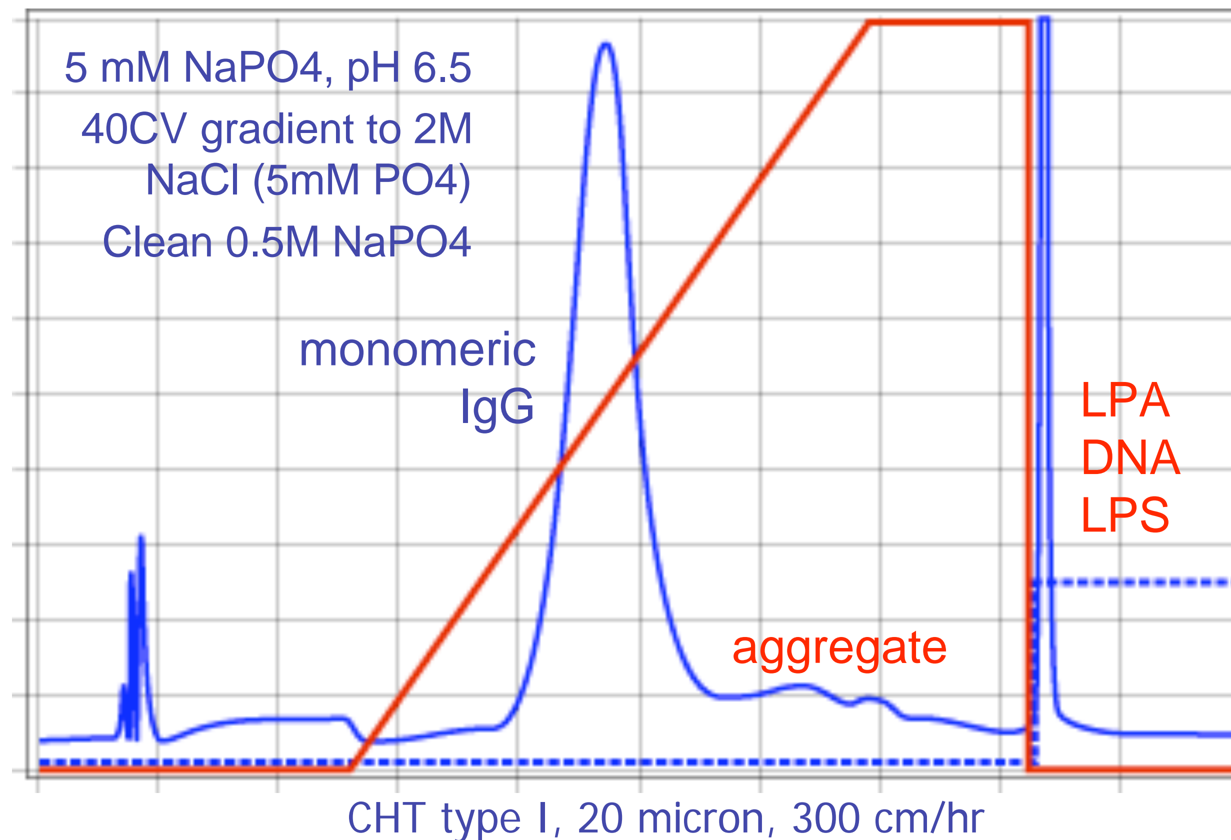
protein A purified IgG1 chimera




CHT type I, 20 micron, 300 cm/hr

# How CHT works with IgG

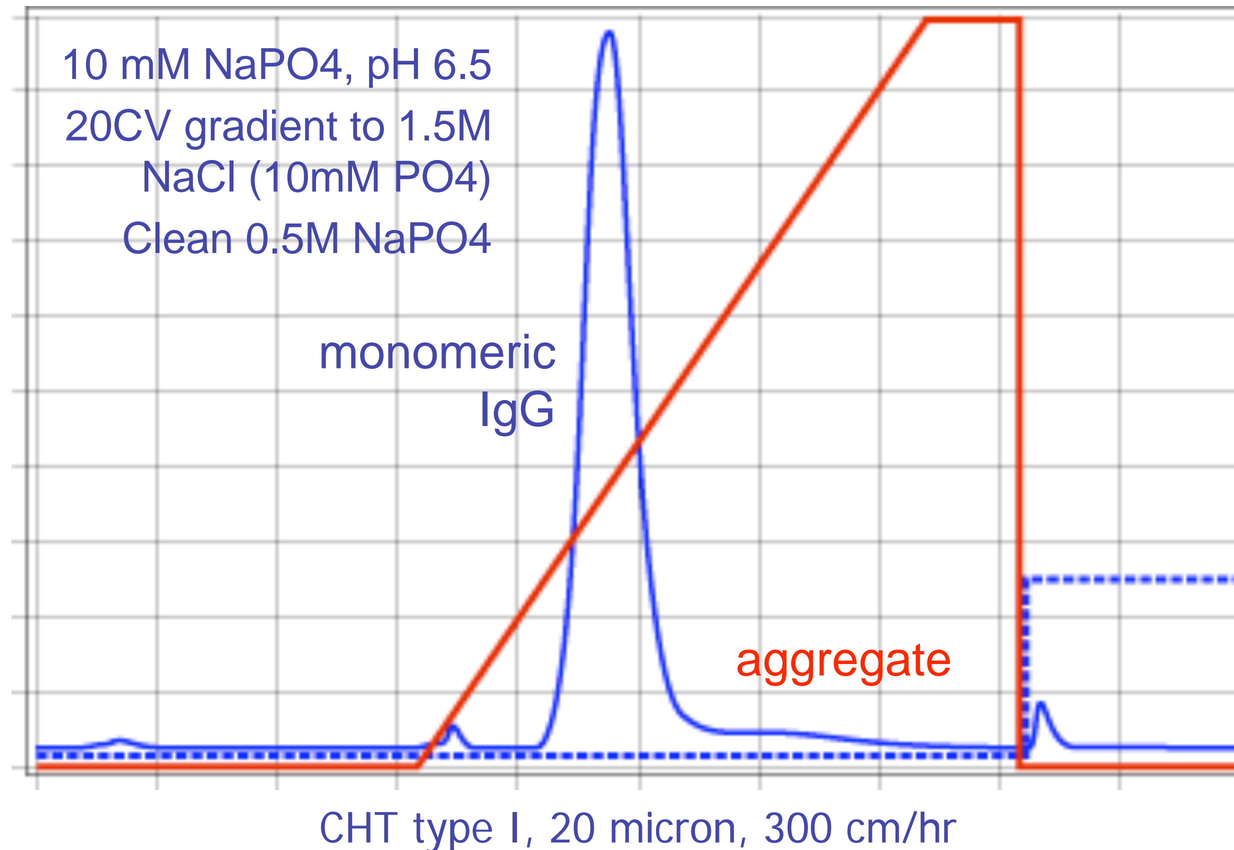
protein A purified human IgG1




CHT type I, 20 micron, 300 cm/hr

# How CHT works with IgG

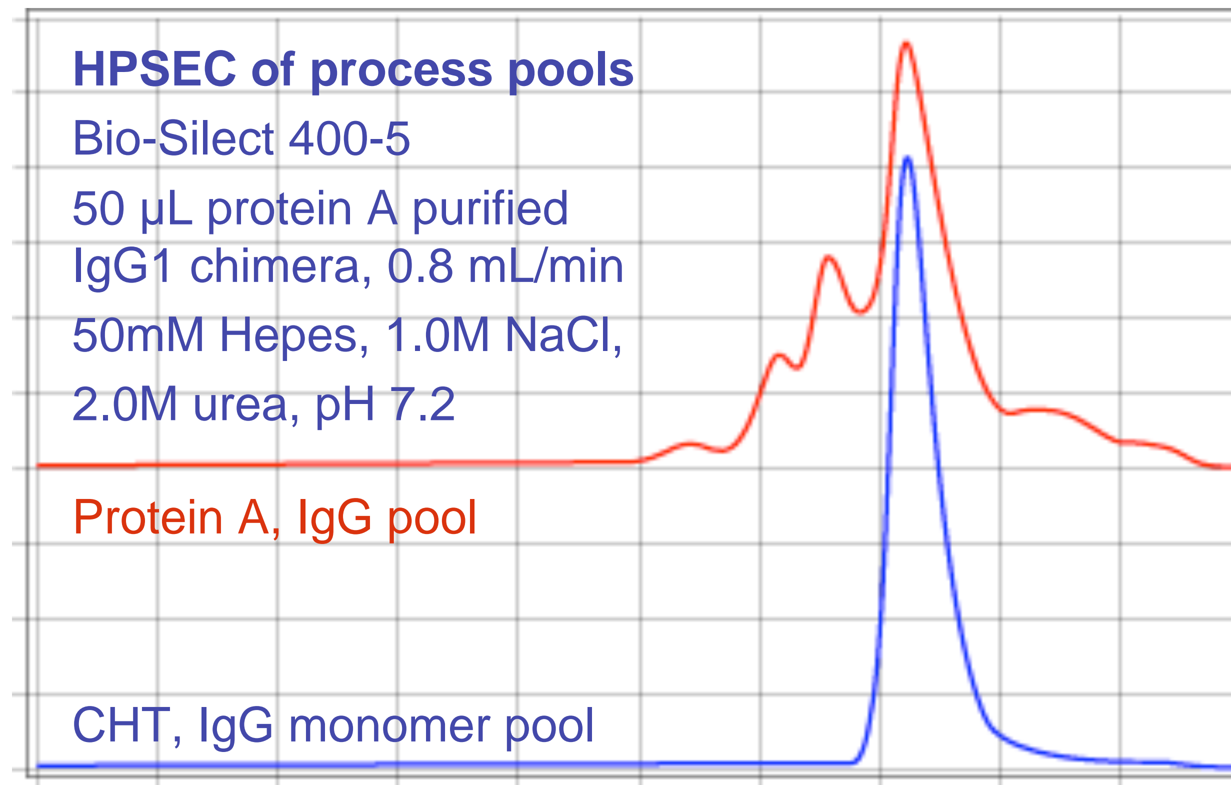
protein A purified mouse IgG1




CHT type I, 20 micron, 300 cm/hr

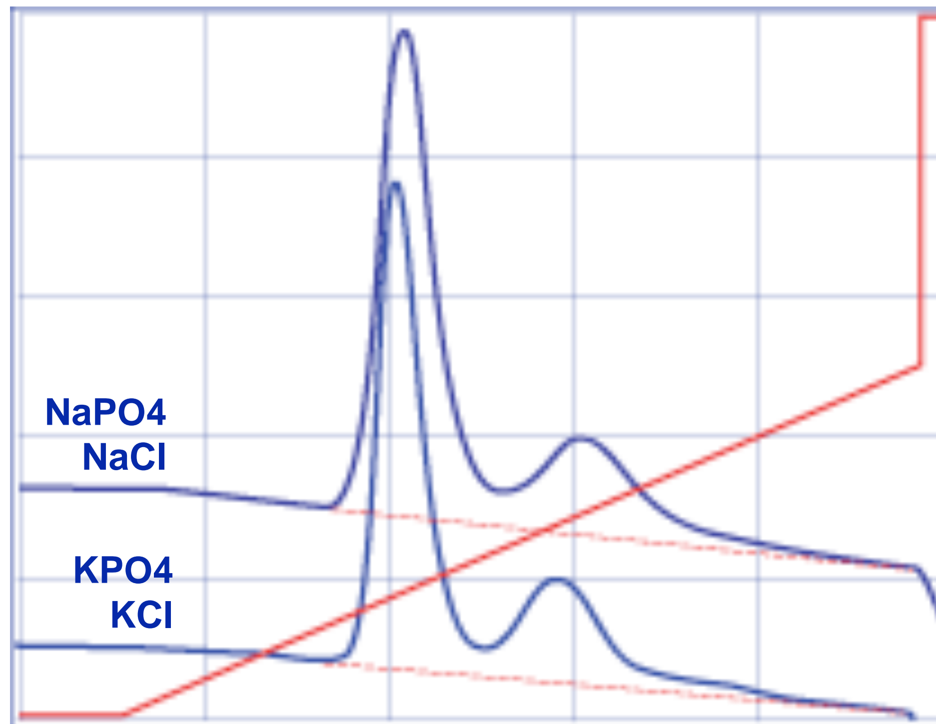
# How CHT works with IgG

Aggregate clearance with NaCl gradients



# How CHT works with IgG

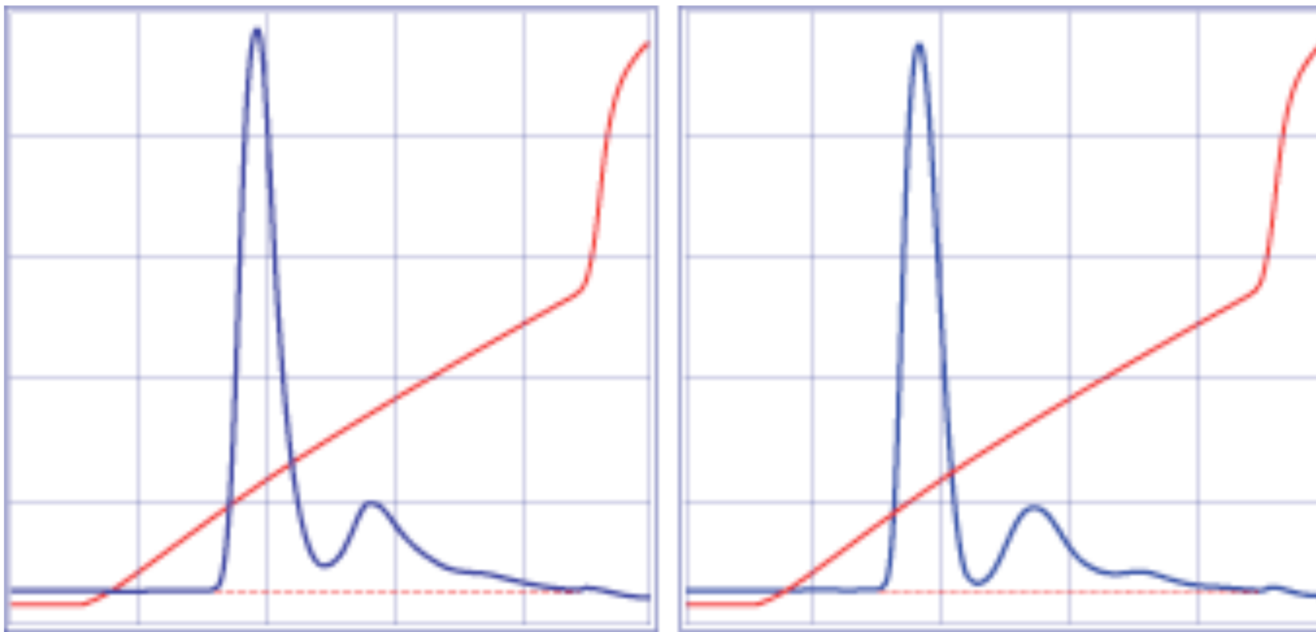
Potassium salts give sharper peaks than sodium salts, but resolution is roughly equivalent.




CHT Type I 20 µm
1 mL, 5 x 50 mm
600 cm/hr,
5 mM PO4 to 5 mM PO4 + 1M Na or KCl, 25 CV

# How CHT works with IgG

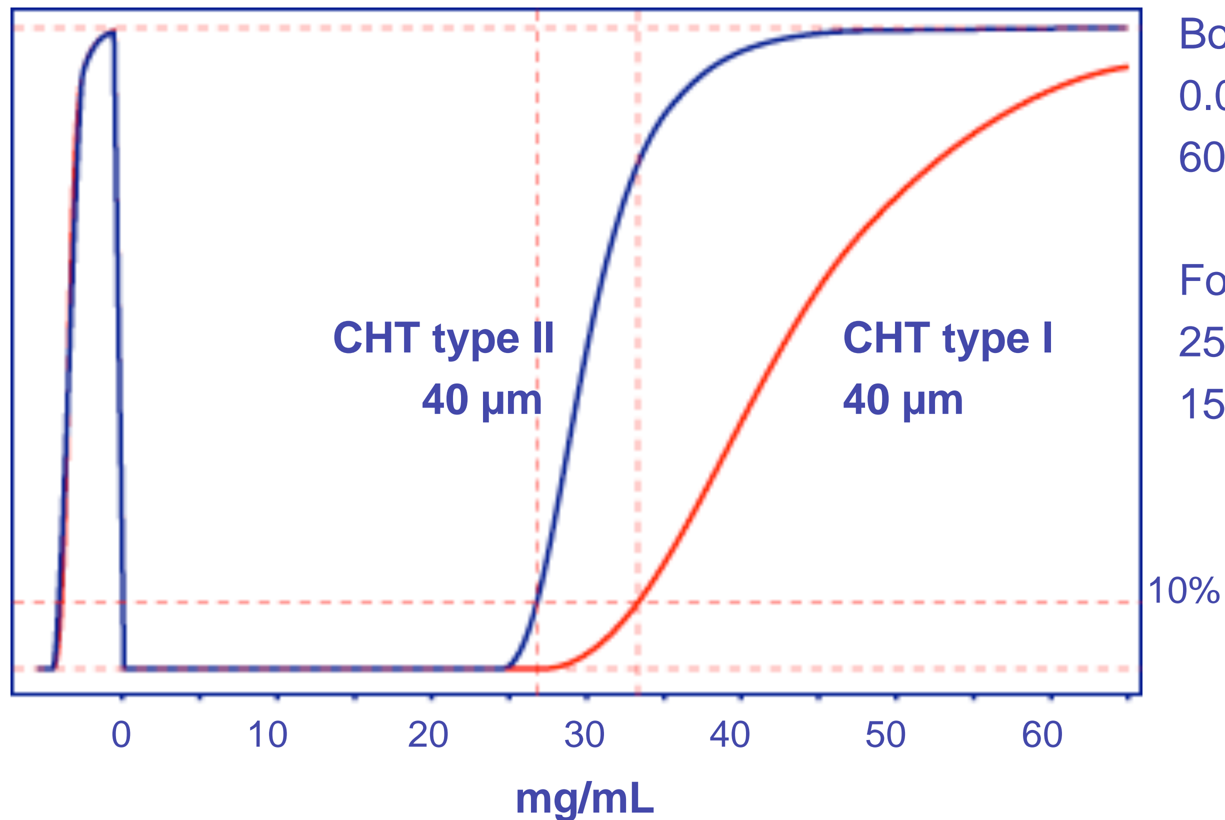
## CHT Type II gives better separation than Type I



20 µm media, 1 mL 5 x 50 mm, 600 cm/hr, 5 mM PO4 to 5 mM PO4 + 1M NaCl, 25 CV

# How CHT works with IgG

## But Type I gives better capacity than Type II




Bovine IgG, 1mg/mL
0.05M MES, pH 6.5
600 cm/hr

For IgG MAbs:
25 - 60mg/mL CHT I
15 - 25 mg/mL CHT II

# CHT method development

**Choice of media**

The higher capacity of Type I makes it more attractive for process applications, but if aggregate removal is a challenge, it may be worthwhile to check Type II.

A 1-2 mL column packed with 20 µm media at 600 cm/hr is effective for initial screening and for modeling separation conditions, however it is too small for the frits on most large scale columns and it gives higher capacity than 40 µm.

Use 40 µm media at 300 cm/hr to characterize capacity and model the process at scales >20 mL.

# CHT method development

**Choice of buffers**

Selectivity is sufficiently similar between sodium and potassium salts that they can be used interchangeably.

Sodium salts are more widely used and more economical.

Potassium salts need to be removed from injectable products however potassium phosphate may be more effective for cleaning CHT, and it is more convenient to prepare at high molar concentrations because of its higher solubility.

# CHT method development

**Sample preparation**

Arginine, glycine, and acetate are all tolerated by CHT as long as a minimum of 5mM phosphate is present. The sample must not contain citrate, EDTA or other strong calcium chelators.

For sample injections of 5 -10% CV, 20-50 mM phosphate and 100 mM NaCl in the sample will be tolerated. Higher salt concentrations may reduce or prevent binding. pH should be at least 6.5.

For large volume sample injections, at least 5mM phosphate in the sample is necessary. pH should be at least 6.5.

# CHT method development

**Initial screening**

**Equilibrate**: with 5 mM Na phosphate, pH 6.7

**Inject**: filtered sample, 5-10% CV

**Wash**: 2-5 CV equilibration buffer

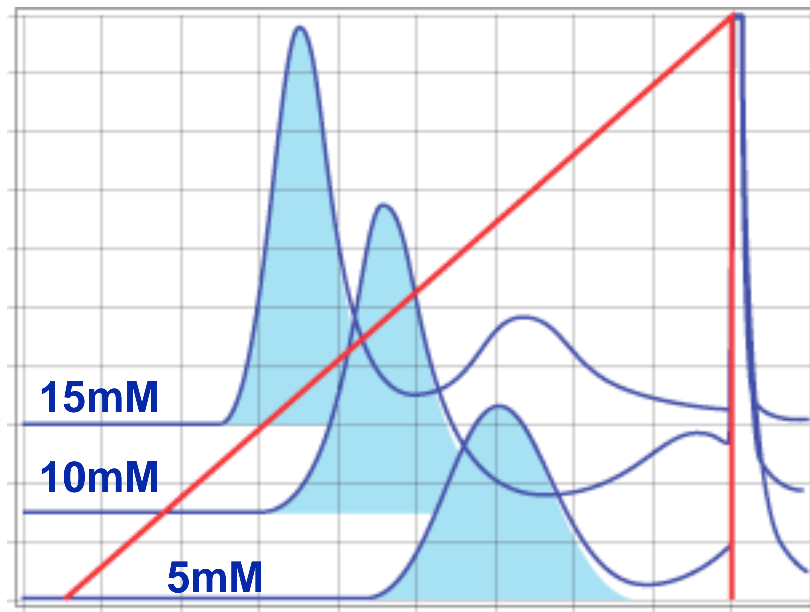
**Elute**: 20 CV linear gradient to 2M NaCl in 5 mM Na phosphate, pH 6.7

**Clean**: 5-10 CV 600 mM K phosphate, pH 6.7

If the antibody does not elute, repeat with 10 mM phosphate in place of 5 mM. If it still does not elute, try 15 mM. Only rare exceptions will require more.

# *The influence of phosphate*

**40 CV linear gradient to 1.0M NaCl at constant phosphate concentrations as indicated**




**protein A purified IgG, CHT type I 20 µm**

Blue areas indicate monomeric IgG, trailing peak is aggregate

Red line indicates NaCl gradient trace

NaCl gradient followed by cleaning with 0.5M phosphate

All experiments at pH 6.5
300 cm/hr

# The influence of phosphate

| Phosphate mM | 5 | 10 | 15 |
|---|---|---|---|
| Protein A ng/mL | n.d. | n.d. | n.d. |
| DNA ng/mL | <1.0 | <1.0 | 3.9 |
| Endotoxin EU/mL | <0.05 | 1.0 | 1.6 |

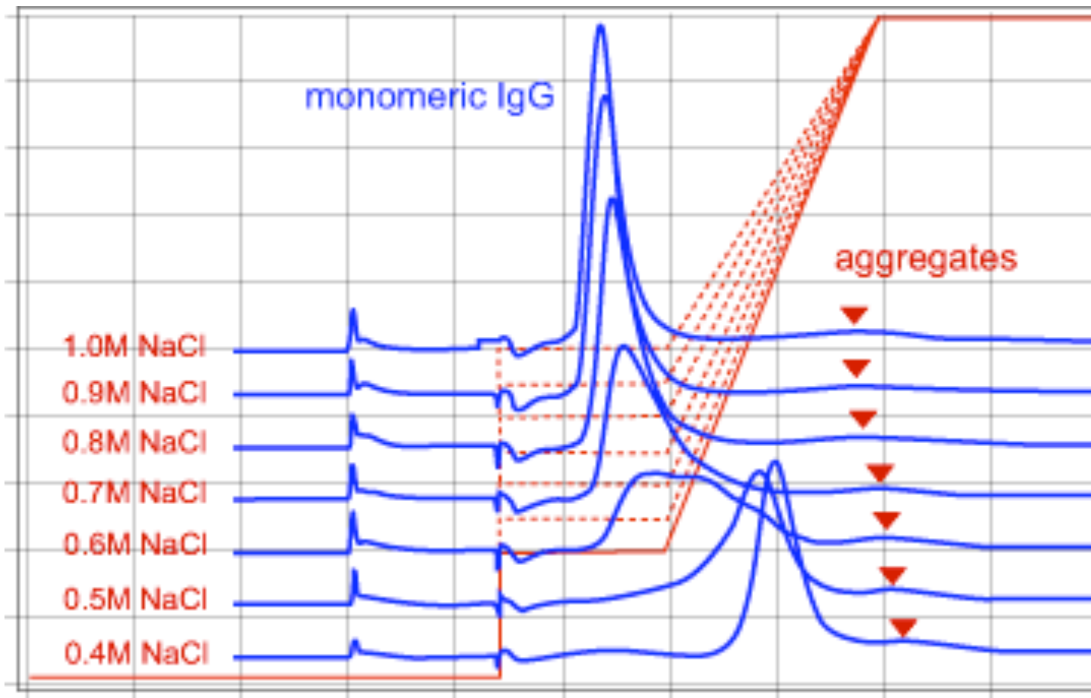
Sample: protein A purified chimeric monoclonal IgG1. 22 ng/mL leached protein A, $2.3 \times 10^3$ ng/mL DNA, $1.9 \times 10^4$ EU/mL endotoxin

Linear detection limit of protein A assay: 0.2ng/mL

All results for the monomeric IgG pool from a sodium chloride gradient to 1.5 M at pH 6.5 with phosphate concentration held at the indicated level. CHT Type I, 40 µm, 300cm/hr.

# Conversion to steps

**Protein A purified human monoclonal IgG1, CHT type I 20 μm**




All experiments in 5mM NaPO4 at pH 7.0, 300 cm/hr

Elution gradients 25CV (step + linear)

Red lines indicate NaCl gradient traces

Red notations indicate step concentration

# CHT method development

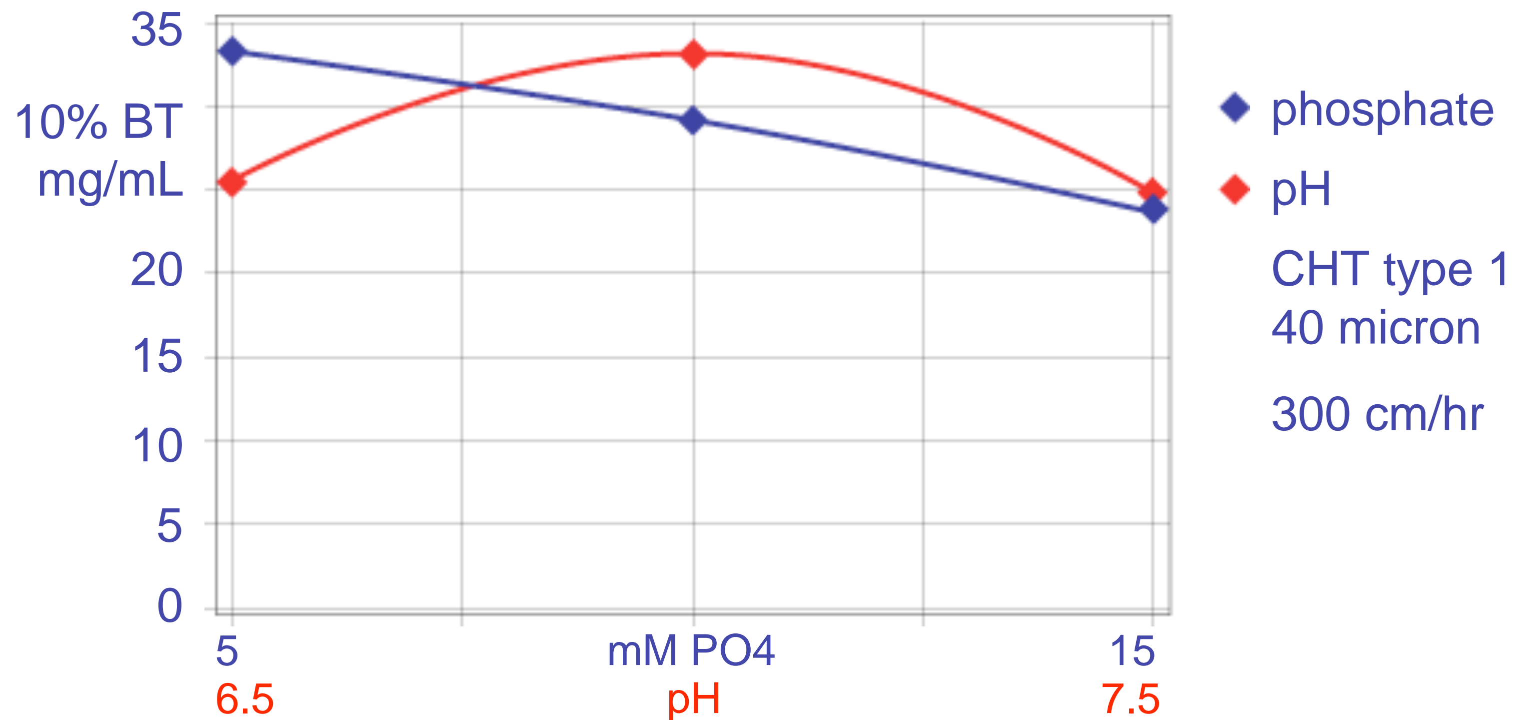
## Capacity

1. Determine the pH that gives the highest binding capacity with a phosphate concentration of 5 mM.* This is about pH 7.0 for most antibodies.
2. Establish capacity tolerance for NaCl. Some antibodies are affected severely, some mildly.

* 5mM phosphate is required to maintain the stability of CHT at pH 6.5; about 2.5 mM at pH 7.5. Use the minimum phosphate concentration because excess phosphate depresses antibody binding capacity as well as removal of DNA, endotoxin, and leached protein A. Operation at pH values below 6.5 is not recommended.

# Capacity versus phosphate and pH

## Dynamic binding capacity, polyclonal human IgG




phosphate experiments conducted at pH 7.0
pH experiments conducted in 5mM phosphate

# 3-Step platform

Elute protein A with 0.1M arginine,* 0.05 M NaCl, pH 3.8**

Hold for virus inactivation

Titrate pH to 7.0** with 1M Tris

EQ strong anion exchanger to 0.05M Tris, 0.05M NaCl, pH 7.0** Apply sample. Collect flow-through

Add 0.5M NaPO4, pH 7.0** to achieve optimal phosphate concentration (1% v:v yields 5 mM)

Conduct virus filtration

Equilibrate CHT with optimal NaPO4, pH 7.0**

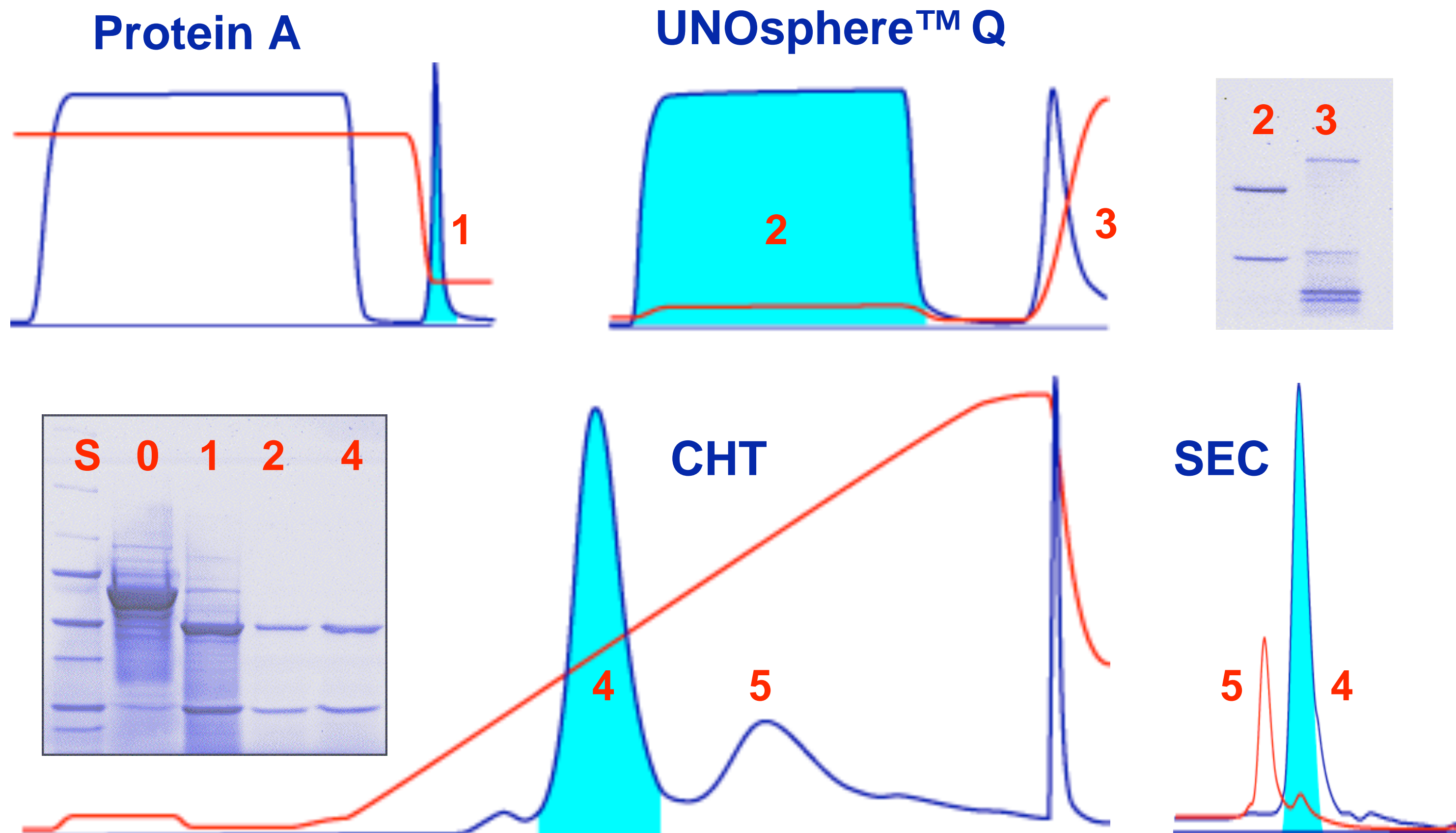
Load, wash, elute under optimized conditions

Concentrate/diafilter to final formulation conditions

* Glycine or acetate can also be used for elution. Citrate degrades CHT and has been shown to reduce leached protein A removal efficiency of anion exchange chromatography.

** Or other pH according to scouting results

# 3-Step platform




Reduced SDS PAGE fractions: S=standards, 0=original material, 1=protein A elution, 2=UNOsphere Q flow-through, 3=UNOsphere Q elution, 4=CHT monomeric IgG pool

# Buffer tips

Avoid anhydrous phosphates. The process of making them anhydrous creates polyphosphates that can affect performance.

If the phosphate level required to achieve the best selectivity is too low to provide adequate buffering capacity, co-formulate with MES, Hepes, Tris, etc., according to the required operating pH.

# Column hygiene

**Clean**: 0.6 M KPhosphate, pH 6.5+

**Sanitize**: 1.0 M NaOH*

>2 hours at 23°C

**Store**: 0.1 M NaOH

* >15,000 hours stability in 1.0 M NaOH

# Column hygiene

CHT binds metals from process solutions, causing discoloration at the top of the column.

These metals may come from buffers and salts, process water, or corroded stainless steel process equipment.

Iron is the most common metal contaminant, producing yellow to brown discoloration.

**First course of action: find the source and eliminate it. Metal contamination affects antibodies and all purification methods.**

Short term fix: try adding 100 mg CHT (type I, 40µm)* per liter of buffer during formulation. incubate 1 hour. Microfilter buffers as usual.

* Suggested starting points. Experiment with quantity and time to accommodate your specific process solutions.

# Recent references

*Hydroxyapatite as a Capture Method for Purification of Monoclonal Antibodies*, P. Gagnon, S. Zaidi, and S. Summers, IBC World Conference and Exposition, San Francisco, Nov. 6-9, 2006.

*Practical Issues in the Industrial use of Hydroxyapatite for Purification of Monoclonal Antibodies* P. Gagnon, 232nd Meeting of the American Chemical Society, San Francisco, Sept. 10, 2006

*The effect of different Hofmeister ions on aggregate removal by hydroxyapatite* P. Gagnon, 232nd Meeting of the American Chemical Society, San Francisco, Sept. 13, 2006

*Monoclonal antibody purification with CHT*, P. Ng, A. Cohen, P. Gagnon, 2006, *Genetic Engineering News*, 26(14) 60

A ceramic hydroxyapatite based purification platform: simultaneous removal of leached protein A, aggregates, DNA, and endotoxins, P. Gagnon, P. Ng, C. Aberrin, J. Zhen, J. He, H. Mekosh, L. Cummings, R. Richieri, S. Zaidi, 2006, *BioProcess International*, 4(2) 50-60.

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